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Enzyme Regulation in Crassulacean Acid Metabolism Photosynthesis[1]

STUDIES ON THE FERREDOXIN/THIOREDOXIN SYSTEM OF *KALANCHOË DAIGREMONTIANA*

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ABSTRACT

Cell-free preparations of the Crassulacean acid metabolism (CAM) plant, *Kalanchoë daigremontiana*, were analyzed for thioredoxins and ferredoxin-thioredoxin reductase. Three distinct forms of thioredoxin were identified in *Kalanchoë* leaves, two of which specifically activated fructose 1,6-bisphosphatase (designated f_1 and f_2) and a third which activated NADP-malate dehydrogenase (thioredoxin *m*). The apparent molecular weight of both forms of thioredoxin *f* was 11,000 and that of thioredoxin *m* was 10,000. In parallel studies, ferredoxin and ferredoxin-thioredoxin reductase were purified from *Kalanchoë* leaf preparations. *Kalanchoë* ferredoxin-thioredoxin reductase was similar to that of C_3 and C_4 plants in molecular weight (31,000) and immunological cross-reactivity. *Kalanchoë* ferredoxin-thioredoxin reductase exhibited an affinity for ferredoxin as demonstrated by its binding to an immobilized ferredoxin affinity column. The purified components of the *Kalanchoë* ferredoxin-thioredoxin system could be recombined to function in the photoregulation of chloroplast enzymes. The data suggest that the ferredoxin/thioredoxin system plays a role in enzyme regulation of all higher plants irrespective of whether they show C_3, C_4, or CAM photosynthesis.

Evidence obtained with C_3 and C_4 plants indicates that light, via Chl, activates key enzymes of photosynthetic and secondary metabolism and deactivates enzymes of degradative pathways (see 7 for references). The antipodal photoregulation of these enzymes allows synthetic and degradative pathways, which share a number of steps, to coexist and operate within the confines of chloroplasts (*e.g.* starch synthesis and degradation). The photoregulation of chloroplast enzymes in C_3 and C_4 plants appears to be effected through several mechanisms, including changes in stromal pH and free Mg^{2+}, alterations in metabolite levels, and the ferredoxin-thioredoxin system.

In the ferredoxin/thioredoxin system, electrons derived from photosynthetic electron transport reduce thioredoxin via ferredoxin and the enzyme FTR[3] (7, 25). The reduced thioredoxin then functions in the modulation of selected chloroplast enzymes. It is now known that there are two types of chloroplast thioredoxins, designated thioredoxin *m* and *f*, which have distinct specificities with respect to 'target' enzymes. Under some conditions, thioredoxin *m* preferentially activates NADP-MDH and thioredoxin *f* activates several enzymes of the reductive pentose phosphate pathway: FBPase, SBPase, NADP-GAPD, PRK, and phenylalanine ammonia lyase. Both types of thioredoxin seem to activate cyanobacterial 3'-phosphoadenosine-5'-phosphosulfotransferase and chloroplast coupling factor, CF_1-ATPase (see references in 7).

Although there is solid evidence that the ferredoxin/thioredoxin system is functional in the photoregulation of C_3 and C_4 photosynthesis, relatively little is known about this system in CAM plants, which are unique in that they fix large quantities of CO_2 at night instead of the day. This nocturnal fixation of carbon is accompanied by the breakdown of storage carbohydrates (such as starch) to C_3 compounds which, when carboxylated, yield four-carbon acids (*i.e.* malate). During the day, the storage carbohydrates are regenerated from the accumulated carbon trapped and stored as four-carbon acids at night (27). According to current ideas, key chloroplast enzymes should be regulated so that synthetic pathways are active in the light and degradative pathways are active in the dark.

In an effort to elucidate the biochemical nature of the photoregulation of enzymes of CAM chloroplasts, we have studied the components of the ferredoxin-thioredoxin system in *Kalanchoë daigremontiana*, an obligate CAM plant. In these studies, we were able to identify in *Kalanchoë* leaves all the components of this system and we now report their isolation and partial characterization.

MATERIALS AND METHODS

Plant Material. *Kalanchoë daigremontiana* Hamet et Perrier and *Sedum praealtum* D.C. were greenhouse grown in University of California mix under natural environmental conditions (3).

Reagents and Proteins. Biochemicals were purchased from Sigma Chemical Co. All other reagents were purchased from commercial sources and were of the highest quality available. Spinach ferredoxin (a gift from R. Chain) was prepared according to Buchanan and Arnon (5). Spinach FBPase was prepared as described by Buchanan *et al.* (6). Corn NADP-MDH was prepared by the method of Jacquot *et al.* (20). A spinach thioredoxin preparation containing both thioredoxins *m* and *f* was obtained by acid and acetone fractionation followed by chromatography on DEAE-cellulose, Sephadex G-75, and hydroxyapatite columns as described by Wolosiuk *et al.* (30). Antibodies prepared against spinach thioredoxin *f*, spinach thioredoxin *m*, and corn FTR were a gift of N. Crawford.

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[3] Abbreviations: FTR, ferredoxin-thioredoxin reductase; ELISA, enzyme-linked immunosorption assay; FBPase, fructose bisphosphatase; NADP-GAPD, NADP-linked glyceraldehyde-3-phosphate dehydrogenase; NADP-MDH, NADP-linked malate dehydrogenase; PRK, phosphoribulokinase; SBPase, sedoheptulose bisphosphatase.

Purification of Thioredoxins *m* and *f*. *Step 1: Extraction of Kalanchoë Leaf Proteins. Kalanchoë* leaves were harvested in the late afternoon to minimize acid content, and immediately frozen in liquid N_2. The frozen leaves could be used directly or stored at −14°C for several months without adverse effects. For extraction of leaf proteins, the frozen leaves (250 g) were combined with approximately 2 L of liquid N_2 and pulverized to a fine powder in a prechilled commercial blender. Any remaining liquid N_2 was allowed to vaporize so that a dry powder remained. The frozen pulverized tissue was slowly added to 750 ml of extraction buffer (100 mM Tris-HCl buffer, pH 7.9, 0.2% [v/v] 2-mercaptoethanol, 6 mM $MgCl_2$, 1% [w/v] PVP-40, and 0.5 mM phenylmethylsulfonyl fluoride) and homogenized for 3 min in a commercial blender. The brei was then filtered through four layers of cheesecloth and the pH of the effluent (generally between 7.2 and 7.5) was adjusted to pH 6.0 with HCOOH. The effluent was centrifuged at 13,000*g* for 30 min and the resulting green precipitate was discarded. The amber supernatant fraction was collected for use below.

Step 2: DEAE-Cellulose Column Chromatography. The amber clarified leaf extract (850 ml) was applied to a DEAE-cellulose column (5 × 25 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 0.1% (v/v) 2-mercaptoethanol (henceforth termed buffer A). After the sample was applied, the column was washed with 500 ml buffer A and thioredoxins were eluted with 900 ml buffer A supplemented with a 0 to 500 mM NaCl linear gradient. During sample application and subsequent wash, 20-ml fractions were collected. Fractions of 4.5 ml were collected during elution by the linear gradient. Column fractions were monitored for $A_{280\ nm}$ and assayed for thioredoxin *m* and *f* activity as described below. This step separated thioredoxin *m* from thioredoxin *f* and also resolved two peaks of thioredoxin *f*. Fractions of each peak were pooled separately and concentrated to 20 ml by ultrafiltration with an Amicon YM-5 membrane.

Step 3. Sephadex G-75 Column Chromatography. The concentrates obtained in step 2 were individually chromatographed on a Sephadex G-75 column equilibrated and developed with buffer A containing 200 mM NaCl. Fractions (3.5 ml) were collected and assayed for activity as described below. Fractions containing thioredoxin activity were pooled for use in experimental studies.

Purification of FTR. *Step 1: Preparation of Leaf Extract.* Cell-free extracts of *Kalanchoë* leaves (250 g) were prepared as described above for the purification of thioredoxins except that the pH of the leaf extract was adjusted to 7.0 before centrifugation. In these studies, we observed that FTR was not stable at pH < 6.0.

Step 2: DEAE-Cellulose Column Chromatography. The green supernatant fraction (850 ml) prepared as described above was fractionated with solid $(NH_4)_2SO_4$. The fraction precipitating between 0 and 90% saturation was collected by centrifugation (13,000*g*, 20 min) and resuspended in 50 mM Tris-HCl buffer (pH 7.9) containing 0.1% (v/v) 2-mercaptoethanol and 3 mM $MgCl_2$ (henceforth called buffer B). This solution was dialyzed overnight *versus* 6 L buffer B and clarified by centrifugation at 13,000*g* for 1 h. The brown supernatant solution (500 ml) was applied to a DEAE-cellulose column (5 × 20 cm) which had been equilibrated beforehand with buffer B. The column was washed with buffer B (400 ml) and eluted first with buffer B (900 ml) supplemented with a 0 to 500 mM NaCl linear gradient and then with 300 ml buffer B supplemented with 500 mM NaCl. During sample application and subsequent wash, 20-ml fractions were collected; during later steps, fraction volume was 4.5 ml. The fractions were monitored for $A_{280\ nm}$ and assayed for FTR and ferredoxin activity by using the assays described below. During this chromatographic step, FTR and ferredoxin were largely separated from each other. Fractions containing peak activity of the each of these two proteins were pooled separately and concentrated by ultrafiltration with an Amicon YM-5 membrane.

Step 3: Bio-Gel A-0.5m Column Chromatography of FTR. The concentrate containing FTR activity (15 ml) was applied to a Bio-Gel A-0.5m column (2.6 × 60 cm) equilibrated and subsequently developed with buffer B supplemented with 200 mM NaCl (henceforth termed buffer C). The NaCl was included to facilitate separation of FTR from ferredoxin. Fractions were collected and assayed for activity and those containing peak activity were combined and concentrated as in step 2. The resulting FTR preparation, which was free of contaminating ferredoxin, thioredoxins, and phosphatases, retained at least 50% of the initial activity after 2 weeks when stored at 5°C.

Purification of Ferredoxin. The ferredoxin concentrate from step 2 above (20 ml) was treated with 1 mg each of ribonuclease (bovine pancreas) and deoxyribonuclease (bovine spleen) overnight at 20°C and the sample subsequently was chromatographed on a Sephadex G-75 column (2.6 × 90 cm) equilibrated and developed with buffer C. Fractions (3.5 ml) were collected and those with the highest *A* at 420 nm were combined and applied directly to a DE52 (Whatman) column (2 × 8 cm) equilibrated with buffer C. The column was eluted with buffer B supplemented with 300 and 500 mM NaCl (50 ml each). Five-ml fractions were collected and analyzed for purity on the basis of their absorption spectra. Fractions with ratios of A_{420}/A_{274} greater than 0.4 were pooled and concentrated as described above for subsequent studies. The ferredoxin preparation so obtained was found to be active in the photoreduction of NADP by spinach thylakoids (see below).

Ferredoxin Affinity Chromatography of *Kalanchoë* FTR. Ferredoxin was bound to CNBr-Sepharose 4B as described in the manufacturer's instructions (affinity chromatography, Pharmacia Fine Chemicals, Uppsala, Sweden). Ferredoxin, 2 mg, purified as described above was dissolved in 4 ml 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl (coupling buffer) and incubated with 0.5 g CNBr-Sepharose for 2 h at 20°C. The buffer was drawn off after the gel had settled and was replaced with 5 ml of 0.2 M glycine, pH 8.0. After 16 h at 4°C, the matrix-ligand complex was washed with 10 ml coupling buffer followed by 10 ml 0.1 M sodium acetate buffer (pH 5.5) containing 0.5 M NaCl. These washes were repeated a second time. The resulting matrix was stable for several months when stored in a sealed chromatography column (0.4 × 10 cm) at 4°C in buffer B supplemented with 500 mM NaCl.

FTR purified through step 3 as described above (1 ml, 0.2 μg protein), was applied to the ferredoxin column (0.4 × 4.5 cm) equilibrated with buffer B. The column was washed with 10 ml buffer B and eluted with a linear NaCl gradient (0–500 mM NaCl) in 30 ml buffer B. Fractions of 0.4 ml were collected. Protein levels in fractions were determined by the microassay method of Bradford (4). FTR was detected immunologically by using ELISA described below.

Assay Procedures. *Assay of FTR.* Previously described procedures were employed for the assay of FTR (30). FTR was assayed by measuring the capacity to promote the photoactivation of chloroplast FBPase or NADP-MDH in the presence of ferredoxin or thioredoxin *f* or *m*, respectively, and thylakoid membranes (all obtained from spinach except for NADP-MDH which was isolated from corn leaves). Thioredoxin *f*-linked FTR activity was routinely assayed at 20°C in Warburg vessels containing in the main compartment 64 μg of spinach chloroplast FBPase, twice-washed spinach thylakoid membrane fragments equivalent to 100 μg of Chl, 50 μg of spinach chloroplast ferredoxin, 60 μg of a spinach preparation containing thioredoxins *f* and *m*, 300 μl of column fraction, and the following (in μmol): Tricine-KOH buffer (pH 8.0), 100; ascorbic acid, 10; 2,6-dichlorophenol indophenol, 0.1; $MgSO_4$, 1.0 (final volume, 1.5 ml). After equilibration for 10 min with N_2, vessels were preincubated for 10 min in the light (1000 μE m^{-2} s^{-1} of 400 to 700 nm light). The reaction was started by adding 6 μmol sodium fructose 1,6-bisP from the side arm and was continued for 45 min under illumination. The reaction was

stopped by the addition of 0.5 ml of 12% (w/v) TCA. The precipitate was centrifuged down and 0.5 ml of the supernatant solution was analyzed for P_i (30).

Thioredoxin *m*-linked FTR activity was determined by using a two-step assay in which aliquots were first preincubated in 1 ml rubber-stoppered Eppendorf centrifuge tubes containing 19 μg corn leaf NADP-MDH, twice-washed spinach thylakoid membrane fragments equivalent to 5 μg Chl, 20 μg of spinach chloroplast ferredoxin, 10 μg of spinach thioredoxin *f* + *m* preparation, 50 μl column fraction, and the following (in μmol): Tris-HCl buffer (pH 7.9), 10; ascorbic acid, 1.0; 2,6-dichlorophenol indophenol, 0.01; 2-mercaptoethanol, 1.4 (final volume, 0.1 ml). After equilibration with N_2 for 10 min, the preincubation solution was incubated for 5 min in the light (1000 $\mu E\ m^{-2}\ s^{-1}$ of 400 to 700 nm light). The reaction was started by injecting 0.05 ml of the preincubation mixture into a nitrogen-saturated reaction mixture containing (in μmol): Tris-HCl buffer (pH 7.9), 100; NADPH, 0.25; 2-mercaptoethanol, 14; oxalacetic acid, 2.5 (final volume, 1 ml). The change in *A* at 340 nm was followed with a Cary model 219 recording spectrophotometer equipped with a sample changer so that multiple assays could be followed simultaneously.

Assay of Thioredoxins. Thioredoxins *f* and *m* were assayed by measuring their capacity to promote the respective DTT-linked activation of spinach chloroplast FBPase or corn leaf NADP-MDH (30). For measuring thioredoxin *f* activity, a modified assay was developed. Thioredoxin *f* was assayed in air at 22°C in 1 × 7.5-cm test tubes containing (in 0.45 ml): FBPase, 3.2 μg protein; fraction, 50 or 100 μl; and the following (in μmol): Tricine-KOH buffer (pH 8.0), 50; $MgSO_4$, 1.5; DTT, 2.5. After the components were preincubated for 10 min, the reaction was started by addition of 0.05 ml of 60 mM sodium fructose 1.6-bisP. The reaction was stopped after 30 min by addition of 2.0 ml of the mixture used for the determination of P_i (30).

Thioredoxin *m* was assayed in air at 22°C by preincubating fractions with partially purified corn leaf NADP-MDH (3.8 μg) in 0.2 ml (final volume) of a solution containing the following (in μmol): Tris-HCl buffer (pH 7.9), 20; and DTT, 2. After a 5-min preincubation, the mixture was transferred to a 1-cm cuvette of 1-ml capacity containing (in 0.75 ml) the following (in μmol): Tris-HCl buffer (pH 7.9), 100; and NADPH, 0.25. The reaction was started by addition of 2.5 μmol oxalacetic acid (in 0.05 ml). The change in *A* at 340 nm was measured in a Gilford model 252 spectrophotometer equipped with a sample changer so that multiple assays could be followed simultaneously.

Assay of Ferredoxin. Ferredoxin was assayed by measuring the capacity to promote the photoreduction of NADP in the presence of spinach thylakoid membranes and ferredoxin-NADP reductase (22) or in purified preparations by its *A* at 420 nm.

Analytical Procedures. Chl was estimated by the method of Arnon (2). Protein was determined by the method of Bradford (4). P_i was measured by a modified Fiske-SubbaRow procedure (30). Concentrations of NaCl in fractions collected during DEAE chromatography were calculated from the conductivity of 1000-fold dilutions of column fractions.

Polyacrylamide gel electrophoresis (nondenaturing) was carried out according to Hedrick and Smith (17) in slab gels (0.75-mm thickness). Procedures of Chua (9) were employed for electrophoresis in SDS-containing polyacrylamide gradient gels (7.5–16%, w/v, acrylamide).

Analytical isoelectric focusing was performed according to Catsimpoolas (8) in vertical 7.5% acrylamide slab gels containing 2% (v/v) ampholytes (1 part ampholytes pH 3.5–10, 4 parts pH 5–8). Samples (100 μl in 12% [w/v] sucrose, 2% ampholytes) were placed in wells and overlaid with 10 μl 5% sucrose. Anode and cathode solutions were 10 mM H_3PO_4 and 20 mM NaOH, respectively. Isoelectric focusing was carried out at constant voltage (400 v) for 16 h and bands were sharpened by 30 min at 800 v. After proteins were fixed by 4 h incubation in 4% (w/v) sulfosalicylic acid and 12% (w/v) TCA, gels were stained for protein by using 27% (v/v) isopropanol, 10% (v/v) acetic acid, 0.04% (w/v) Coomassie blue R-250, 0.5% (w/v) $CuSO_4$. Gels were destained after 4 h with 12% isopropanol, 7% acetic acid, and 0.5% $CuSO_4$ followed by 7% acetic acid, 5% (v/v) methanol. For electroblotting, gels were prewashed for 2 min in 1 L H_2O.

Molecular weight of FTR was estimated by chromatography on a calibrated Bio-Gel A-0.5m column (2.6 × 60 cm) equilibrated and developed with 50 mM Tris-HCl buffer containing 0.1% (v/v) 2-mercaptoethanol and 200 mM NaCl. Molecular weights of thioredoxins were determined by gel filtration on a calibrated Sephadex G-75 column (2.6 × 90 cm), equilibrated and developed with Buffer A supplemented with 200 mM NaCl.

Other Procedures. *ELISA.* Minimum quantities of antigenic protein were estimated by using the ELISA procedures of Weeden and Gottlieb (28). Protein samples, containing between 10 and 200 ng protein, were bound to wells of ELISA Cuvette paks (Gilford Instrument Co, Oberlin, OH) by incubation at 37°C for 3 h in 100 μl of 0.1 M sodium carbonate buffer (pH 9.6). Wells were washed three times with 100 μl wash buffer (20 mM sodium phosphate buffer, pH 7.3, 0.14 M NaCl, 0.05% Tween-20); then 100 μl of binding buffer (wash buffer supplemented with 0.1% globulin-free BSA and 1 mM EDTA) containing an appropriate amount of antibody was added to each well. After 1 h at 37°C, wells were washed with wash buffer as before. Binding buffer containing peroxidase-conjugated anti-rabbit IgG goat IgG (Sigma Chemical Co.; diluted 1:400) was added to wells and incubated for 30 min at 37°C. Wells were washed four times as above and a peroxidase reaction mixture containing 32 mM sodium citrate buffer (pH 4.0), 0.01% H_2O_2, and 0.2 mM 2,2′-azino-di(3-ethylbenzthiazoline) sulfonic acid. After 30 min, the reaction was stopped by the addition of 0.2 ml 0.1 M HF. The *A* at 415 nm was read on a eia automatic analyzer (model PR-50). Quantity of antigenic protein was estimated by comparison to standards.

RESULTS AND DISCUSSION

Identification of *Kalanchoë* Thioredoxins *f* and *m*. We first investigated whether leaves of CAM plants contain thioredoxins analogous to those of C_3 and C_4 plants surveyed thus far. In our initial survey, we were able to detect in leaf extracts of *K. daigremontiana* and *Sedum praealtum* both thioredoxin *f* and *m* by measuring their capacity to promote the respective DTT-dependent activation of FBPase and NADP-MDH. After identifying both of these thioredoxins in CAM preparations, we attempted to compare them with thioredoxins of other photosynthetic organisms. Because of the ease of propagation and culture, *K. daigremontiana* was used as the source for thioredoxins and related proteins in subsequent studies.

In leaves from other species, thioredoxins *m* and *f* can be separated by DEAE-cellulose chromatography (11, 29). When *Kalanchoë* thioredoxin preparations were chromatographed on DEAE-cellulose under our usual conditions (*i.e.* at pH 7.9), we observed a single peak of thioredoxin activity that showed both thioredoxin *f* and *m* activities. However, by reducing the pH of the elution buffer to 5.5, *i.e.* near the isoelectric point of other chloroplast thioredoxins characterized in this respect (18, 25), three peaks of thioredoxin activity were resolved (Fig. 1). One peak which passed freely through the column was selective for activation of NADP-MDH (designated thioredoxin *m*) whereas two subsequent peaks of approximately equal activity selectively promoted the activation of FBPase. The peak of activity eluted by the wash buffer was designated thioredoxin f_1 and the one eluted by salt was designated thioredoxin f_2. Interestingly, the *Kalanchoë* *f*- and *m*-type thioredoxins could not be resolved by chromatography on Sephadex G-75 or hydroxyapatite under conditions successfully used for the spinach equivalents.

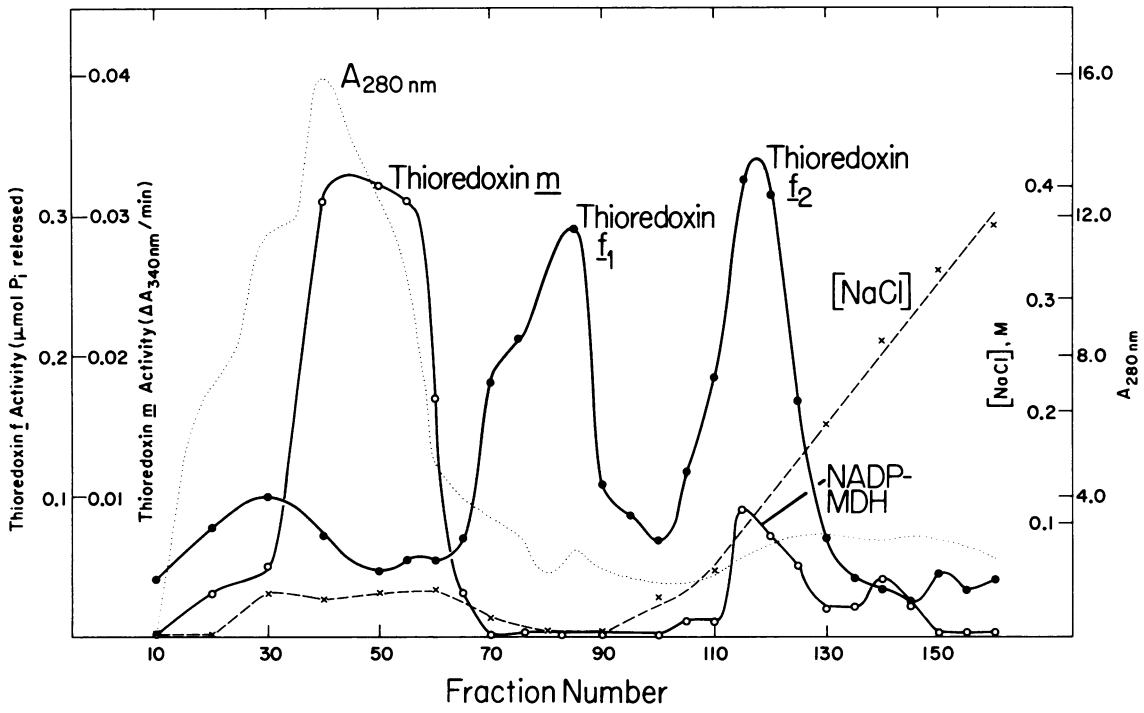

FIG. 1. Separation of *Kalanchoë* leaf thioredoxins by DEAE-cellulose chromatography.

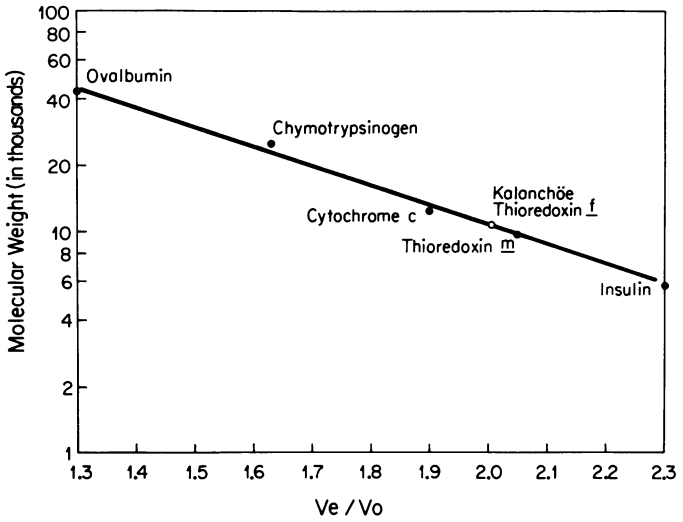

FIG. 2. Molecular weight of *Kalanchoë* thioredoxins as determined by Sephadex G-75 column chromatography.

The molecular basis for the multiple forms of *Kalanchoë* thioredoxin *f* has not been established. One possible explanation is that of an artifact due to overloading of the DEAE-cellulose column used for their resolution. This, however, does not appear to be the case. Enzymes which co-migrated with *Kalanchoë* thioredoxin f_2 during chromatography (SBPase and NADP-MDH) did not contaminate fractions containing thioredoxin f_1 (data not shown). Furthermore, a repeat chromatography of thioredoxins f_1 and f_2 at pH 5.5 on DEAE-cellulose revealed that both forms migrated as before, *i.e.* thioredoxin f_1 was not adsorbed by the matrix whereas thioredoxin f_2 required low salt to elute. Similar chromatographic behavior was observed in preparations containing DTT-reduced thioredoxins, indicating the oxidation/reduction state of thioredoxins was not influencing chromatographic behavior. Finally, degradation due to proteases also appears to be excluded: equal activities and similar apparent mol wt of both *f*-type thioredoxins were observed in preparations prepared in the presence or absence of phenylmethylsulfonyl fluoride. The data thus collectively indicate that *Kalanchoë* thioredoxins f_1 and f_2 are different proteins and are not artifacts of the purification procedure.

Multiple species of thioredoxins *m* and *f* have been reported in leaves of corn and sorghum (18, 21) and unique thioredoxin species have been observed in different barley tissues (seeds, etiolated leaves, green leaves) (11). Schürmann (25) reported that two species of chloroplast thioredoxin *m*, differing by the presence of an additional lysine at the NH_2 terminus, are present in spinach. In addition to chloroplast thioredoxins, apparent cytoplasmic equivalents have also been reported in spinach leaves (10, 29). However, attempts to purify and characterize the cytoplasmic thioredoxins have not been highly successful and this problem warrants further study.

Properties of Thioredoxins. After identification and resolution of the multiple forms of *Kalanchoë* thioredoxin, we characterized these proteins with respect to mol wt and initiated experiments to elucidate the isoelectric point and cross-reactivity of these proteins to antibodies raised against chloroplast thioredoxins from other sources. These studies were hindered by the instability of *Kalanchoë* thioredoxins. In contrast to *m*-type thioredoxins from other plants, *Kalanchoë* thioredoxin *m* was highly unstable, retaining less than 10% of its initial activity after 24 h at 4°C under conditions used routinely for other thioredoxins (Tris-HCl buffer, pH 7.9). Although more stable than thioredoxin *m*, both thioredoxins f_1 and f_2 lost approximately 15% of initial activity daily under similar conditions. In addition, all three thioredoxins were sensitive to freezing. It was only through the use of low pH (5.5) and 2-mercaptoethanol in elution buffers that we were able to stabilize *Kalanchoë* thioredoxins to an extent suitable for their partial characterization.

Like thioredoxins from other organisms, *Kalanchoë* thioredoxins are low mol wt proteins. Based on Sephadex G-75 sieve chromatography, the apparent mol wt of thioredoxin *m* was 10,000 and that of the two thioredoxin *f* species was 11,000 (Fig. 2). In these studies, we noted that the apparent mol wt of *Kalanchoë* thioredoxin *f* varied with the ionic strength of the buffer. When

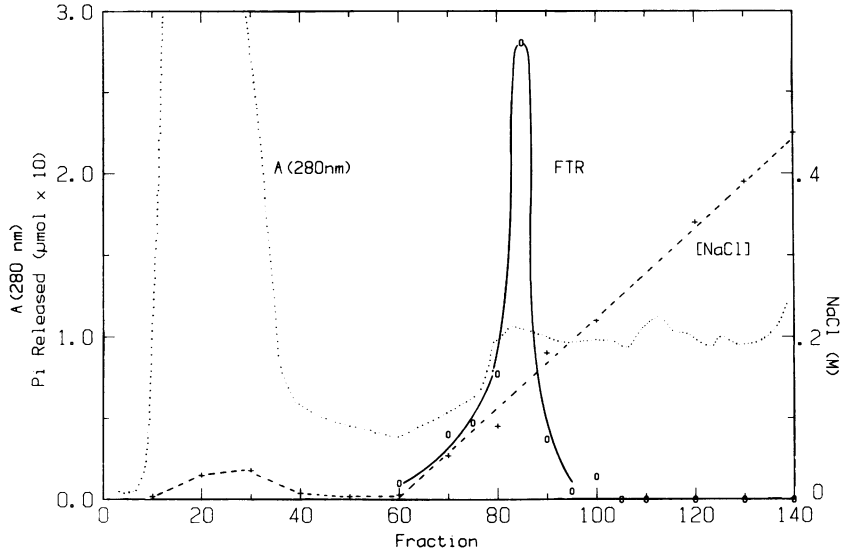

FIG. 3. DEAE-cellulose column chromatography profile of *Kalanchoë* FTR.

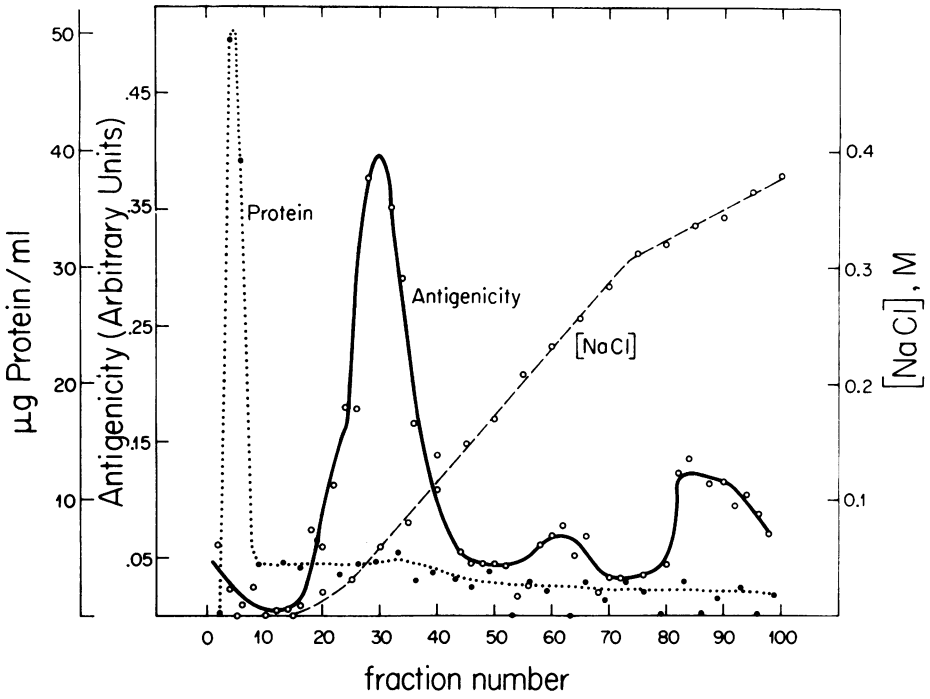

FIG. 4. Elution profile of *Kalanchoë* FTR after ferredoxin affinity chromatography.

chromatographed in low ionic strength buffer (NaCl omitted), *Kalanchoë* thioredoxin *f* migrated as a 20,000- to 25,000-D protein (data not shown), suggesting the formation of a dimer similar to that described for thioredoxins of spinach under these conditions (26). Alternative explanations for this chromatographic behavior can be tendered, however. The inflated mol wt of thioredoxin *f* during molecular sieve chromatography at low ionic strength may stem from its interaction with the column matrix (see manufacturer's instructions) or with other proteins in the relatively crude preparations. In support of this later premise, thioredoxin *f* was a frequent contaminant of *Kalanchoë* FBPase and SBPase after gel filtration chromatography in low salt buffers (19). This is in accord with recent work indicating that spinach thioredoxin *f* has a high affinity for FBPase, requiring salt for dissociation (24).

Mol wt of *Kalanchoë* thioredoxins similar to those of spinach were also indicated by Western blot analysis (23) following SDS-polyacrylamide gel electrophoresis. By using antibodies raised against spinach chloroplast thioredoxin *f*, we observed in preliminary experiments a single antigenic band in *Kalanchoë* thioredoxin f_1 and f_2 preparations which co-migrated with spinach thioredoxin *f* (data not shown). Similar analyses employing antibodies against spinach leaf thioredoxin *m* indicated that the *Kalanchoë* thioredoxin *m* preparation contained an antigenic protein which, like spinach, showed a mol wt of 10,000 (data not shown).

Western blot procedures also allowed us to probe isoelectric focusing gels for thioredoxins. Preliminary experiments indicated that *Kalanchoë* thioredoxin *m* has an isoelectric point of 5.0, similar to its equivalent in spinach and corn (18, 25). The isoelectric point of *Kalanchoë* thioredoxin f_1 was found to be pH 6.1 whereas two forms of thioredoxin f_2 were identified with respective pI values of 6.2 and 6.9. In these experiments, the thioredoxin *m*

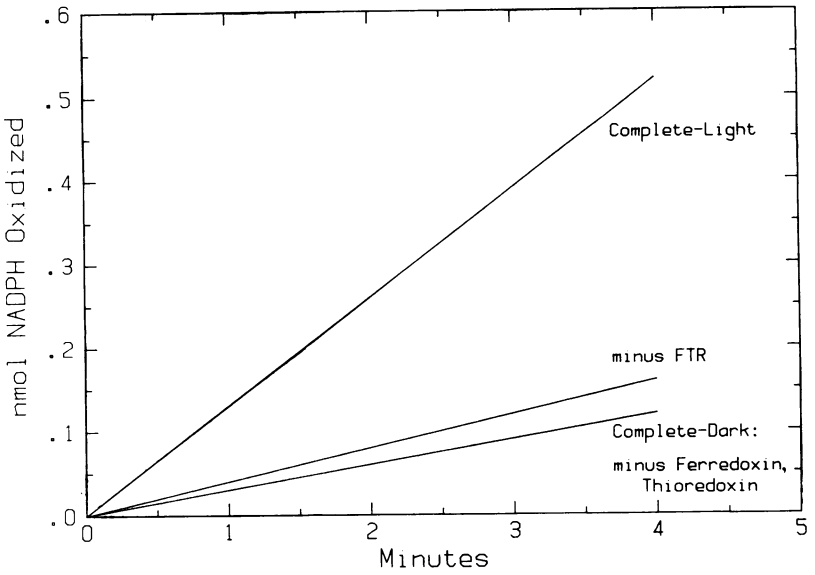

FIG. 5. Photoactivation of *Kalanchoë* NADP-MDH by a thioredoxin *m*-linked ferredoxin/thioredoxin system. *Kalanchoë* FTR, 20 μg, was present as indicated. Other conditions were as described in "Materials and Methods" for the assay of thioredoxin *m*-linked FTR.

Table I. *Photoactivation of Spinach FBPase by a Reconstituted Kalanchoë Ferredoxin/Thioredoxin System*

The reaction was carried out in Warburg-Krippahl vessels. The complete system contained in the main compartment 48 μg of spinach FBPase; freshly prepared, twice-washed spinach chloroplast membrane fragments equivalent to 100 μg of Chl; *Kalanchoë* leaf ferredoxin (15 μg); *Kalanchoë* FTR (40 μg); *Kalanchoë* thioredoxin f_2 (1.4 μg); and the following (in μmol): Tricine-KOH buffer (pH 8.0), 100; $MgCl_2$, 1. Sodium fructose 1,6-bisP (6 μmol) was added to the side arm. Final volume, 1.5 ml. Temperature, 20°C.

Treatment	Relative Activity
	%
Light	
Complete	100[a]
Minus thioredoxin	20
Minus ferredoxin	17
Minus FTR	6
Dark	
Complete	25

[a] Activity, 5 nmol P_i released/min.

and *f* preparations were found to be immunologically distinct.

Identification of FTR in *Kalanchoë* Leaf Extracts. In contrast to most heterotrophic cells, the reduction of thioredoxins in chloroplasts has been shown to require reduced ferredoxin rather than NADPH (7). This ferredoxin-linked reduction of thioredoxins is catalyzed by FTR, an enzyme that has been isolated and partially characterized from spinach and corn (13, 18, 25). By using assay components purified from spinach, we were able to identify FTR in *Kalanchoë* leaf preparations following fractionation by DEAE-cellulose chromatography (Fig. 3). The resulting preparation was further purified by molecular sieve chromatography. In these studies, no ferralterin activity was detected in extracts of *Kalanchoë* leaves (*cf.* 14).

Kalanchoë FTR was found to share several physical properties with its C_3 and C_4 counterparts. As determined by gel filtration chromatography, *Kalanchoë* FTR has an apparent mol wt of 31,000, slightly smaller than its 32,000- to 38,000-D counterparts in spinach (13) and corn (18). Because our preparation was only partially purified, we were not able to discern whether *Kalanchoë* FTR has a constituent chromophore (*cf.* 25).

In these experiments, we observed that FTR preparations frequently contained contaminating ferredoxin following molecular sieve chromatography in low ionic strength buffers. This observation suggested formation of a complex between ferredoxin and FTR as found recently for FTR from corn leaves under these conditions (12). To test this premise, we prepared a ferredoxin affinity column by cross-linking homogeneous *Kalanchoë* ferredoxin to CNBr-Sepharose 4B. As shown in Figure 4, *Kalanchoë* FTR, monitored by its reaction with corn FTR antibody in the ELISA assay, was selectively adsorbed to the immobilized ferredoxin, requiring salt for dissociation and elution. Greater than 80% of the antigenic activity applied to the ferredoxin column was recovered in a single peak which in Western blot analysis was similar to FTR from other species (data not shown). These results thus indicate that *Kalanchoë* ferredoxin and FTR form a complex under low ionic strength conditions. It remains to be established whether FTR similarly forms a complex with thioredoxins. During these studies we noted that *Kalanchoë* ferredoxin resembled other higher plant ferredoxins in mol wt, M_r = 10,000 as determined by Sephadex G-75 column chromatography, and absorption spectrum (data not shown) (*cf.* 16).

A point not yet covered pertains to the thioredoxin specificity of *Kalanchoë* FTR. In spinach leaves, thioredoxin *f*- and *m*-linked FTR activities appear to reside on a single protein (13). In contrast, it has been suggested that the multiple FTR species found in corn leaves may have specificity with respect to thioredoxin *f* and *m* (12). In the current study, thioredoxin *f*-linked FTR was easily identified, but thioredoxin *m*-linked FTR activity was not initially apparent. After considerable difficulty, we were able to detect thioredoxin *m*-linked FTR activity in FTR preparations (purified through the DEAE-cellulose chromatography step by the capability to activate FBPase) through the use of stringent anaerobic conditions and of 2-mercaptoethanol in the preincubation and reaction mixtures. The *Kalanchoë* FTR preparation mediated a thioredoxin- and ferredoxin-dependent photoactivation of NADP-MDH (Fig. 5). However, due to high background activity, low sensitivity of the assay, and low activity of the preparation, we were not able to discern clearly the thioredoxin *m*-linked FTR profile after DEAE-cellulose chromatography carried out under conditions that resolve different corn FTR species (N. Crawford, unpublished results).

Reconstruction of the Ferredoxin-Thioredoxin System of *Kalanchoë* Leaves. This laboratory has proposed that thioredoxins func-

tional in enzyme photoregulation are reduced in illuminated chloroplasts via ferredoxin and FTR (7). In the experiments described above, we have shown above all components of the ferredoxin-thioredoxin system to be present in *Kalanchoë* leaves (*i.e.* ferredoxin, FTR, and thioredoxins *m*, f_1, and f_2). However, those experiments were based on heterogeneous assays involving spinach thioredoxins and ferredoxin or DTT. It, therefore, is of interest to know whether the components of the *Kalanchoë* ferredoxin-thioredoxin system can be reassembled to form a system functional in enzyme photoregulation. To this end, we partially purified ferredoxin, FTR, and thioredoxin *f* from *Kalanchoë* leaves in a concerted manner and tested them for their capability to photoactivate FBPase. As shown in Table I, the results of this experiment were positive: when the components of the ferredoxin/thioredoxin systems were appropriately recombined, chloroplast FBPase was activated photochemically. In this experiment, as in previous ones, we found it advantageous to use spinach thylakoid membrane fragments as photochemically active chloroplasts are difficult to isolate from *Kalanchoë*.

Concluding Remarks. The results presented above show that all components of the ferredoxin/thioredoxin system are present and potentially functional in the regulation of photosynthetic enzymes in the CAM plant, *K. daigremontiana*. Furthermore, aside from differences in physical properties, the components of the system (ferredoxin, FTR, thioredoxins *f* and *m*) seem to be similar to those of C_3 and C_4 plants, indicating that the basic design of this system has been maintained during evolution. It thus now seems likely that the ferredoxin/thioredoxin system plays a role in enzyme regulation in all types of higher plants, irrespective of whether they show C_3, C_4, or CAM photosynthesis. The succeeding communication (19) identifies enzymes that are under regulation of this system in the CAM plant, *K. daigremontiana* (*cf.* 1, 15).

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